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# Molecular Phylogeny of the Lady Fern Genus *Athyrium* in Japan Based on Chloroplast *rbcL* and *trnL–trnF* Sequences

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Phylogenetic relationships in *Athyrium* and *Cornopteris* were deduced from two chloroplast DNA fragments, *rbcL* and *trnL* 5′exon–*trnF*, of 32 species, 2 varieties, 3 putative hybrids of *Athyrium*, three taxa of *Cornopteris*, and five outgroups. *Athyrium* is paraphyletic, and the *Athyrium–Cornopteris* complex comprises five clades. Clade I, the most basal, comprises *A. niponicum*, *A.* (= *Anisocampium*) *sheareri*, and *A.* (= *Kuniwatsukia*) *cuspidatum.* Clade II includes *A. distentifolium* and *Cornopteris*. All species of clades III and IV are diploids, while most species of clade V are polyploids. The parentage of the putative hybrids and of species of hybrid origin were also suggested. The results were compared to previous major classifications based on morphology.

Key words: *Athyrium*, hybrid, molecular phylogeny, *rbcL*, *trnL–trnF*

*Athyrium* Roth is a worldwide genus of about 200 species. Most of the species are distributed in the northern hemisphere, especially in eastern and southeastern Asia as well as in the Himalaya and adjacent mountain chains; comparatively few species are found in tropical and southern Africa or in South America, and very few are in Europe (Kramer & Kato 1990). Approximately 37 species, 1 subspecies, 6 varieties, 9 forms, and 74 putative hybrids occur in Japan. Most taxa are in the southwestern part of the country (Iwatsuki *et al.* 1992). Several Japanese species groups within the genus have been recognized and revised based on morphology, including the *A. yokoscense*, *A. otophorum*, *A. vidalii*, *A. iseanum*, and *A. filix-femina* groups (Tagawa 1933, Kurata 1961, Serizawa 1981). Wang (1997, 1999) revised the Chinese species of *Athyrium*, classifying them into 14 sections. Among the species recognized in those studies, 20 taxa are common in Japan. However, the monophyly of each group and the phylogenetic relationships among the groups have not been examined. The very large number of interspecific hybrids also poses a problem in understanding the taxonomy of the genus, because the hybrids can obscure the circumscription of species. Unfortunately, only two genetic studies on putative hybrids or hybrid species have been undertaken (Kurihara *et al.* 1996, Terada & Takamiya 2006).

In addition to the paucity of knowledge on

intrageneric subdivisions, the generic circumscription of *Athyrium* is still controversial. The genus was recently revised (Kramer & Kato 1990) to include small allied genera such as *Anisocampium* C. Presl, *Pseudocystopteris* Ching, *Cystoathyrium* Ching, and *Kuniwatsukia* Pichi Sermolii. In molecular phylogenetic studies of the lady fern group including *Athyrium* and allied genera (Sano *et al.* 2000, Wang *et al.* 2003), *Athyrium* (Kramer & Kato 1990) was suggested to be paraphyletic, because the species of *Cornopteris* Nakai were nested within the *Athyrium* clade.

The goal of the present study was to elucidate the phylogenetic relationships between species of *Athyrium sensu* Kramer & Kato (1990) mainly from Japan, and also to examine the intergeneric relationship between *Athyrium* and *Cornopteris*. Although the molecular studies of Sano *et al.* (2000) and Wang *et al.* (2003) proposed to resolve the intergeneric relationships in the lady fern group, the number of species of *Athyrium* sampled in those studies was insufficient to determine the intrageneric subdivisions of *Athyrium*. Our ingroup sample set comprised 37 taxa of *Athyrium* and three taxa of *Cornopteris*, which represent about 64% of the taxa of *Athyrium*, except hybrids, in Japan. The phylogenetic analysis was based on a combined dataset of two chloroplast regions, the *rbcL* gene and the region from *trnL* 5′ exon to *trnF*. The *rbcL* gene alone has been widely used to analyze many fern lineages (Hasebe *et al.* 1993, 1994, 1995, Pryer *et al.* 1995, Wolf *et al.* 1994, Gastony & Ungerer 1997, Sano *et al.* 2000, Little & Barrington 2003, Lu *et al.* 2007), but the combination with *trnL–trnF* was expected to improve resolution, particularly for studying intrageneric relationships (Schneider *et al.* 2004, Geiger & Ranker 2005, Lu *et al.* 2005, Driscoll & Barrington 2007). Our findings fit a revised taxonomy of the genus *Athyrium* that combines elements of all previous systems into a phylogenetically meaningful classification.

## Materials and Methods

*Taxon sampling*

A total of 137 plants from 43 taxa was collected for DNA extraction. For about half of the taxa, we determined chloroplast DNA sequences from multiple samples. GenBank sequences of non-Japanese and uncollected Japanese taxa were also added to the DNA sequence dataset. The sources of the materials and sequences are listed in Appendix 1. The ingroup taxa of the *rbcL* dataset comprised 32 species, two varieties, and three putative hybrids of *Athyrium*, as well as two species and one hybrid of *Cornopteris*, representing all previously recognized major groups in Japan. Three species of *Deparia* Hooker & Greville and two of *Diplazium* Swart were chosen to serve as an outgroup, based on previous phylogenetic analyses (Sano *et al.* 2000, Wang *et al.* 2003). The freshly collected materials were preserved in silica gel until DNA extraction.

*DNA extraction, PCR amplification, and sequencing*

The total DNA was extracted from dried leaf samples using the method of Doyle & Doyle (1987), although some samples were extracted using DNeasy Plant Mini Kit (Qiagen, Tokyo, Japan). The PCR primers aF and cR of Hasebe *et al.* (1994) were used to amplify *rbcL* fragments, and specific primers rbcL2F (5′CCCCCTGCTTATTCCAAAAC3′) and rbcL2R (5′TTCCGGCGTGTATATGATCC3′) were designed as internal primers for sequencing. The region from *trnL* (UAA)5′exon to *trnF* (GAA), later called *trnL–F*, was amplified with forward primer trnLF2F (5′ATGAATTTCGGGCGATGAG3′), which was designed for this study, and with primer *f* of Taberlet *et al.* (1991). The PCR products were purified using the Geneclean III kit (Qbiogene, Irvine, CA, USA) after electrophoresis in 1% aga-

rose gel and used as templates for direct sequencing. Sequencing reactions were carried out with a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). All sequencing reactions were processed using either ABI 310 or ABI 377XL automated sequencers (Applied Biosystems). A total of 91 new DNA sequences were deposited in GenBank as part of this study (Appendix 1).

Sequence fragments were analyzed using the Sequencing Analysis v5.2 (Applied Biosystems) and assembled by use of SeqScape v2.5 (Applied Biosystems). The corrected consensus sequences were then automatically aligned on ClustalX 1.81 (Thompson *et al.* 1997) followed by manual adjustment using BioEdit (Hall 1999).

*PCR-SSCP analysis*

Parentage and hybridity were tested on the putative hybrids or hybrid species incorporated into phylogenetic analyses. The partial nuclear *PgiC* gene was amplified with primer set 15F and 16R of Ishikawa *et al.* (2002) for putative hybrids, hybrid species, and their hypothesized parents. To survey intraspecific genetic variation, multiple samples were examined for hypothesized parental species in this analysis. The PCR products were analyzed using the single-strand conformation polymorphism (SSCP) method following the procedure of Watano *et al.* (2004).

*Phylogenetic analysis*

A separate phylogenetic analysis was conducted for each dataset (*rbcL*, *trnL–F*, and the combined dataset). Neighbor-joining (NJ) analysis and maximum parsimony (MP) analysis were performed with PAUP* 4.0b10 (Swofford 2002), and Bayesian phylogenetic analysis was performed with MrBayes ver.3.1.2 (Huelsenbeck & Ronquist 2001). All of the phylogenetic analyses were conducted with indels excluded. A partition homogeneity test as implemented in PAUP* was performed to estimate incongruent length differences between the two single sequence datasets.

The NJ tree was constructed with genetic distance set according to Kimura's two-parameter method (Kimura 1980) and with bootstrapping of 1,000 replicates. MP trees were calculated with the following options: heuristic search mode, tree bisection-reconnection (TBR) branch swapping, MULtrees option on, and collapse zero-length branch off. Branch support was estimated by bootstrap analysis (Felsenstein 1985) with full heuristic searches, 1,000 replicates. In Bayesian analysis, each region was assigned its own model of nucleotide substitution as determined by the Akaike information criterion (AIC) in MrModeltest 2.0 (Nylander 2004). For the combined dataset, we ran a mixed-model analysis, allowing each region to evolve under its own best-fit model. Posterior probabilities of generated trees were approximated using a Markov chain Monte Carlo (MCMC) algorithm with four incrementally heated chains for 1 million generations and sampling trees every 100 generations. A 50% majority-rule consensus tree was calculated to obtain topology with average branch lengths as well as posterior probabilities for all resolved nodes. We considered values greater than 85% to indicate strong support for common ancestry.

## Results

*Sequence characteristics*

We determined DNA sequences of both cpDNA regions from multiple samples (two to six per taxon) for about half of the taxa. There was no intraspecific sequence variation, except in *Cornopteris christenseniana* and *Athyrium tashiroi* (one substitution in *trnL–F*). The alignment of the 47 *rbcL* sequences (three from GenBank) of *Athyrium* and allied genera consisted of 1200 characters, of which 140 (11.7%) were variable and 100 (8.3%) were parsimony-informative. The

sequence of the *trnL–F* region of *A. frangulum* f. *viride* could not be determined, nor could the *trnL* intron sequences of the three taxa from GenBank used in the *rbcL* dataset (*A. distentifolium*, *A. filix-femina*, and *A. spinulosum*). Therefore, 43 sequences were included in the *trnL–F* dataset. The *trnL–F* sequences varied from 694 base pairs (bp) in *Cornopteris christenseniana* to 761 bp in *A. niponicum*. The alignment is available upon request from the corresponding author. The aligned sequence of *trnL–F* resulted in a matrix of 816 characters, of which 245 (30%) were variable and 190 (23.3%) were parsimony-informative. The aligned combined *rbcL* and *trnL–F* matrix of 43 taxa consisted of 2016 characters; 379 (18.8%) of these were variable and 289 (14.3%) were parsimony-informative. The best-fit model for each data partition is SYM+I+G for *rbcL* and GTR+G for *trnL–F*.

*Separate phylogenetic analysis*

Phylogenetic analysis was conducted for each dataset employing NJ, MP, and Bayesian methods. The MP method recovered 1557 shortest trees of 246 steps (CI = 0.736; RI = 0.871) for the *rbcL* dataset, and 1185 shortest trees of 380 steps (CI = 0.700; RI = 0.869) for *trnL–F* dataset. These tree reconstruction methods generated mostly congruent topologies for each dataset. Thus, Bayesian trees with the support of NJ bootstrap, MP bootstrap, and Bayesian posterior probabilities are shown in Figs. 1 and 2 as examples.

In the *rbcL* analysis, there are five major clades, each with different statistical support (Fig. 1). Clade I was positioned at the most basal position of the tree and comprised three species: *Athyrium niponicum*, *A. sheareri*, and *A. cuspidatum*. Clade II comprised *A. distentifolium* and *Cornopteris*, and was sister to the group of Clades III, IV, V, with some species not included in any clades. Clade III received strong support in all analyses. Clade IV was weakly supported by NJ and Bayesian analyses, and was not supported by MP. The large clade V, a diverse evolutionary group, was supported only in Bayesian analysis (PP = 67%). The relationships between clades III, IV, and V were not resolved.

All phylogenetic hypotheses of the *trnL–F* dataset recovered the same five clades with the same memberships as a result of the *rbcL* dataset (Fig. 2). Each clade was supported as monophyletic in all three analyses with moderate or strong support. Support of the five clades was higher in *trnL–F* than in *rbcL*. The ILD test indicated no significant conflict between the two datasets (p = 0.243), indicating the combinability of the datasets.

*Combined phylogenetic analysis*

The parsimony analysis of the combined *rbcL* and *trnL–F* data recovered 102 shortest trees of 568 steps (CI = 0.717; RI = 0.875). The tree obtained from the Bayesian analysis (shown in Fig. 3) resulted in nearly the same topology as the NJ and MP analyses of the combined dataset (not shown). Consistent with both individual dataset topologies, the combined analysis recovered five major clades (clades I–V), all of which were moderately or strongly supported in all analyses. The relationships among clades were the same as those in the separate dataset analyses, and clades III, IV, and V were not resolved even in the combined dataset. Using the combined dataset, we recognized two subclades (Va and Vb) within a large clade V with strong support. The relationships of *Athyrium atkinsonii* and *A. rupestre* to clades III, IV, and V were varied in each analysis, and thus were not included in the clades defined here. The result from the combined analysis is taken here as the best estimate of the phylogenetic relationships of the genus, because it is the best summary of the data to date. The discussion is therefore based on the phylogeny obtained from this analysis unless otherwise noted.

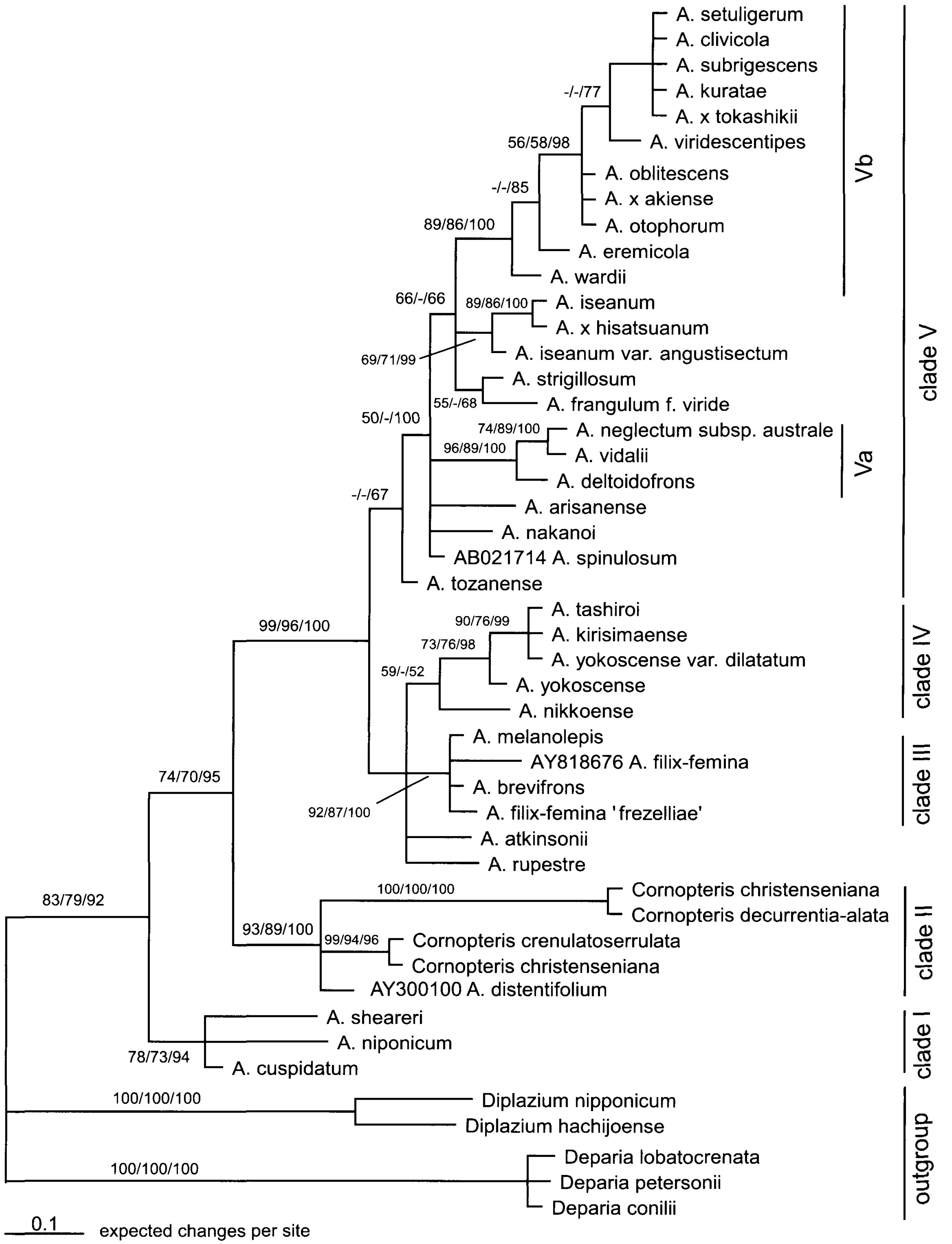


FIG. 1. Phylogenetic tree based on the *rbcL* dataset using a Bayesian analysis. Measures of support are given at the nodes: NJ bootstrap (BS)/MP bootstrap (BS)/Bayesian posterior probabilities (PP). Support values under 50 are shown as hyphens (-).

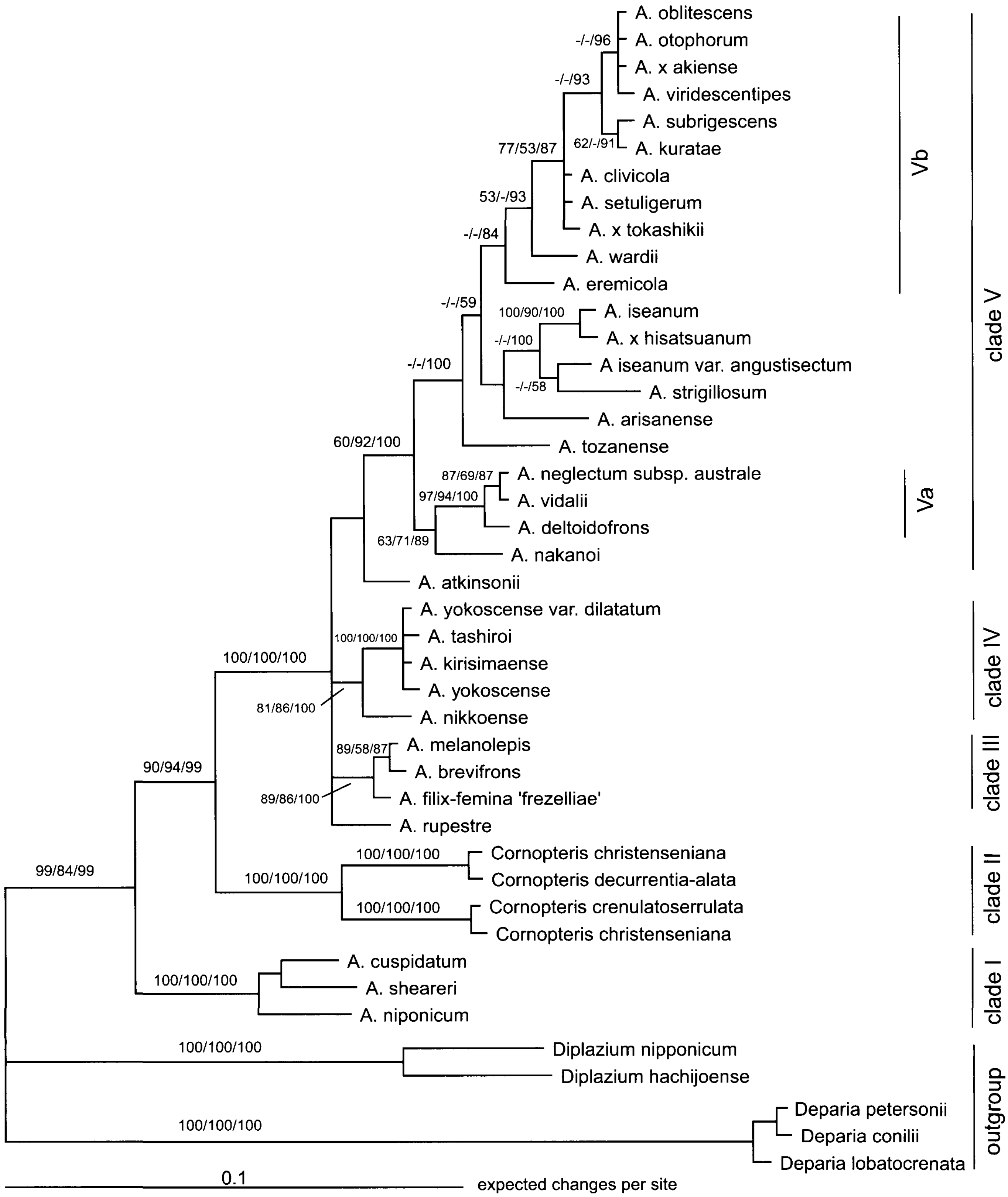


FIG. 2. Phylogenetic tree based on the *trnL–F* dataset using a Bayesian analysis. Measures of support are given at the nodes: NJ bootstrap (BS)/MP bootstrap (BS)/Bayesian posterior probabilities (PP). Support values under 50 are shown as hyphens (-).

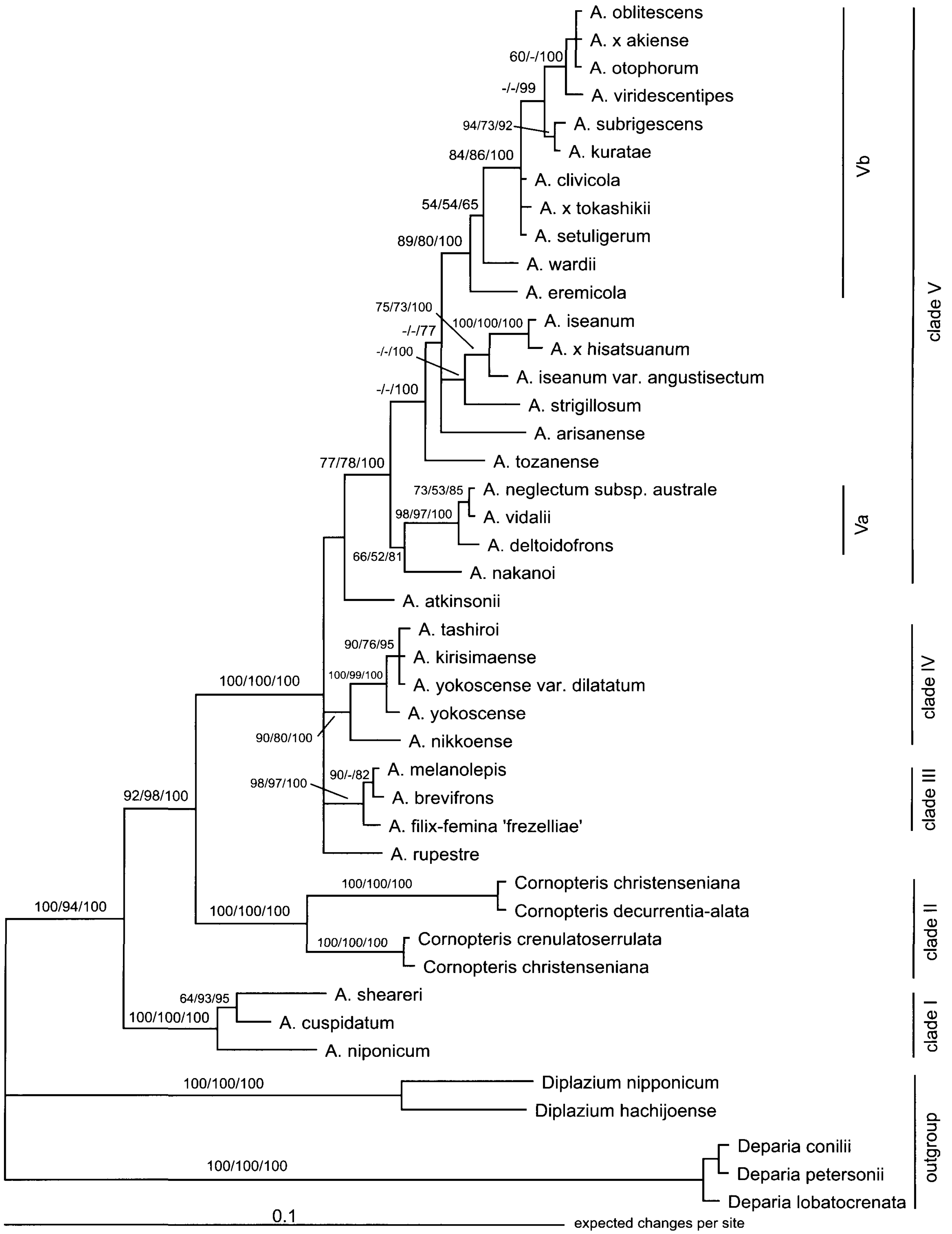


FIG. 3. Phylogenetic tree based on the combined dataset using a Bayesian analysis. Measures of support are given at the nodes: NJ bootstrap (BS)/MP bootstrap (BS)/Bayesian posterior probabilities (PP). Support values under 50 are shown as hyphens (-).

*Analysis of putative hybrids and hybrid species*

We include the following three putative hybrids and two hybrid species of *Athyrium* in the dataset of chloroplast phylogenetic analysis: *A.* ×*akiense* (*A. eremicola* × *A. otophorum*), *A.* ×*tokashikii* (*A. wardii* × *A. clivicola*), *A.* ×*hisatsuanum* (*A. iseanum* × *A. clivicola*), *A. oblitescens* (*A. otophorum* × *A. wardii* and *A. otophorum* × *A. clivicola*), and *A. setuligerum* (*A. clivicola* × *A. iseanum*). The chloroplast DNA sequence of each plant was identical to that of either of hypothesized parents. PCR-SSCP of the nuclear single-copy gene (*PgiC*) for these hybrids and taxa of hybrid origin also supported their hypothesized parentage (Fig. 4). For example, *A.* ×*akiense* (lane 11, Fig. 4) showed overlapping band pattern of *A. eremicola* (lane 1) and *A. otophorum* (lane 2), suggesting that the hypothesized parentage was correct.

## Discussion

Phylogenetic relationships in *Athyrium* and *Cornopteris* were deduced from two chloroplast DNA fragments, *rbcL* and *trnL* 5′exon to *trnF*. The results show that *Athyrium* is paraphyletic and that the *Athyrium–Cornopteris* complex comprises five major clades (I, II, III, IV, and V).

Clade I consists of three species in which *Athyrium niponicum* is the sister taxon to *A. sheareri* and *A. cuspidatum*. The placement of this clade as the most basal in the *Athyrium* phylogenetic tree agrees with the results of previous studies (Sano *et al.* 2000, Wang *et al.* 2003). *Athyrium sheareri* and *A. cuspidatum* were formerly treated as members of distinct genera, *Anisocampium* and *Kuniwatsukia*, respectively, showing that the clade is a morphologically diverse group. In comparison with other species of *Athyrium*, *Athyrium sheareri* has an unusual combination of morphological characters, including a long creeping rhizome, pinnate fronds with a chartaceous texture, and orbicular sori (Sano *et al.* 2000). A creeping rhizome is also present in *A. niponicum. Athyrium cuspidatum*, however, has an erect or ascending rhizome. Although this clade is strongly supported, no synapomorphic morphological characters are known.

Our *rbcL* tree shows that *Athyrium distentifolium* and *Cornopteris* are monophyletic,

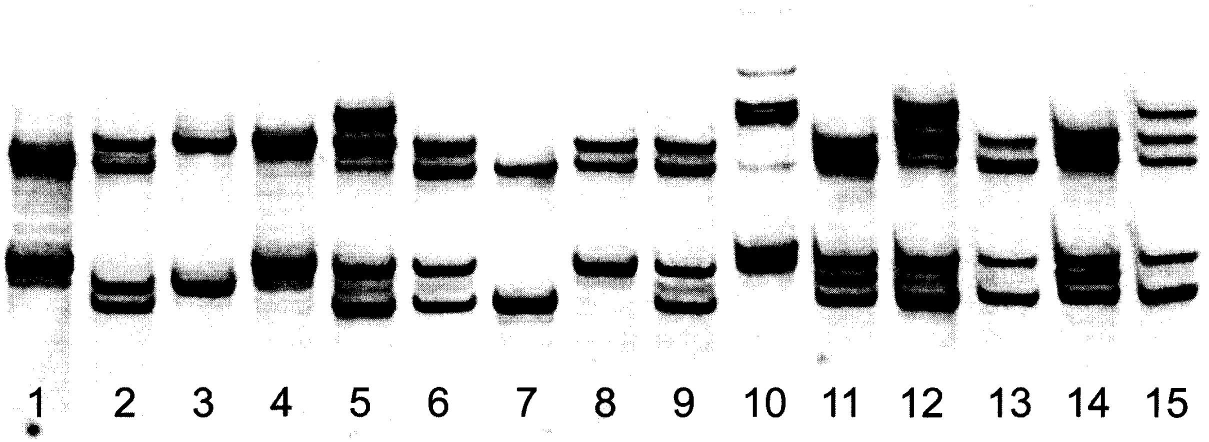


FIG. 4. PCR-SSCP band pattern of partial *PgiC* gene. Lane (1) *Athyrium eremicola*, (2, 3) *A. otophorum*, (4) *A. wardii*, (5, 6) *A. clivicola*, (7) *A. iseanum*, (8, 9) *A. vidalii*, (10) *A. deltoidofrons*, (11) *A.* ×*akiense* [*A. eremicola* × *A. otophorum*], (12) *A.* ×*tokasikii* [*A. wardii* × *A. clivicola*], (13) *A.* ×*hisatsuanum* [*A. iseanum* × *A. clivicola*], (14) *A. oblitescens* [*A. otophorum* × *A. clivicola* or *A. wardii*], (15) *A. setuligerum* [*A. clivicola* × *A. iseanum*].

although the phylogenetic relationship is not yet understood (Fig. 1). *Athyrium distentifolium* has a worldwide distribution and is characterized by circular to elliptic sori, with irregular filaments making up a rudimentary indusium when young, but soon obscured as the sori grow (Iwatsuki *et al.* 1992, McHaffie 2005). In comparison, *Coryopteris* is a small Asian genus with nine species, defined by the corniculate base of the pinnae and pinnules and the exindusiate sori (Kato 1979), while its other morphological characters are similar to those of *Athyrium*. Clade II, therefore, can be recognized as a group with by the synapomorphic characteristic of exindusiate sori on mature

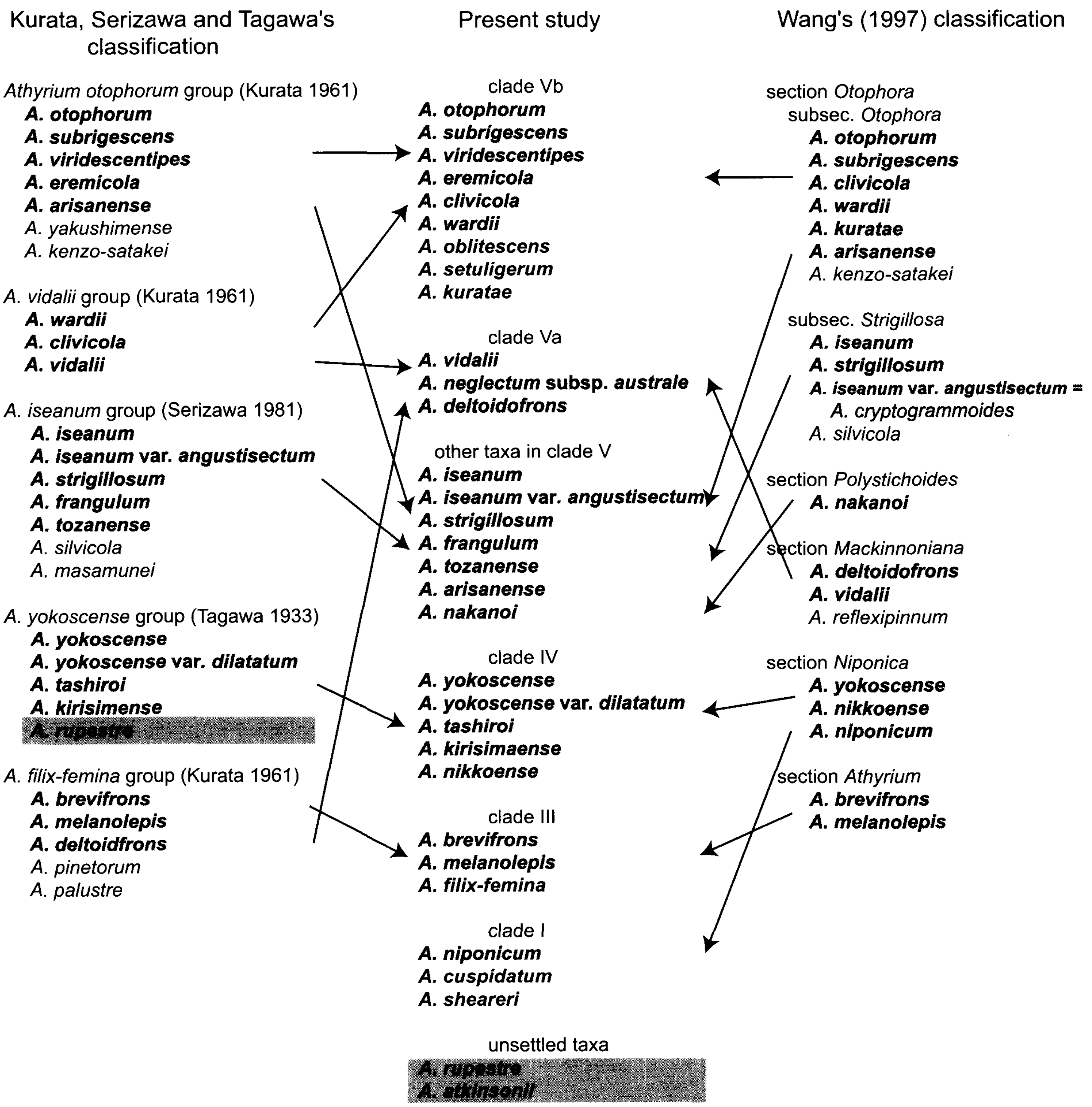


FIG. 5. Comparison of the previous classification (species commonly found in Japan were selected) with the one proposed in the present study. Taxa in boldface represent species examined in this study. The lightly shaded boxes show the correspondence between previous taxonomic groups and clades in our phylogenetic tree. The darkly shaded boxes show species with uncertain positions.

leaves.

The nested position of *Cornopteris* in the tree of *Athyrium* was suggested by previous molecular analyses (Sano *et al.* 2000, Wang *et al.* 2003), and confirmed in the present study based on a wider taxon sampling and longer DNA sequences. Some hybrid taxa between *Athyrium* and *Cornopteris* have been proposed (Kurata 1963, 1965, Hirabayashi 1970, Nakaike 1992). Serizawa (1981b) treated *Cornopteris* as a subgenus of *Athyrium* based on the occurrence of these hybrids. These observations are concordant with the close alliance between *Athyrium* and *Cornopteris* in our analysis. In this study we sampled two specimens of *C. christenseniana.* Park & Kato (2003) confirmed this taxon to be an interspecific triploid hybrid of diploid *C. crenulatoserrulata* and tetraploid *C. decurrenti-alata.* One of our samples of *C. christenseniana* was grouped with *C. crenulatoserrulata* and another was grouped with *C. decurrenti-alata* (Fig. 3), confirming the reciprocal occurrence of hybridization as suggested by Park (2002).

To prevent a paraphyletic *Athyrium*, *Cornopteris* should be included within *Athyrium*, or clades I and II should be treated as genera separate from *Athyrium*. If the latter, then additional studies are desireable to clarify the delimitation of clades I and II. First, the type species of the genus *Anisocampium*, *A. cumingianum* C. Presl was not included in the present study. Because *A. cumingianum* is characterized by goniopteroid venation, which is not shared by *A. sheareri*, the addition of *A. cumingianum* to clade I should be tested. Secondly, Wang (1997) reported that three Chinese species of *Athyrium*, *A. wallichianum, A. dissitifolium* and *A. exindusiatum*, have exindusiate sori. Their phylogenetic relationship to *A. distentifolium* and *Cornopteris* is critical for diagnosing clade II.

The remainder of the members of *Athyrium* form a monophyletic clade and contain several intrageneric subgroups based on morphological characters (Tagawa 1933, Kurata 1961, Serizawa 1981, Wang 1997). Figure 5 illustrates the correspondence of the morphological groups with the clades resolved in this study. Kurata (1961) considered that the *A. otophorum* group, the *A. vidalii* group, and the *A. iseanum* group were closely related to one another, based on the frequent hybridization among them. Our phylogenetic analyses support that opinion because all members of the three groups examined in this study are included in clade V. Furthermore, clade V also contains *A. deltoidofrons* of the *A. filix-femina* group *sensu* Kurata (1961). Interestingly, all members of clade V are polyploids (4*x* or 6*x*) except *A. frangulum* f. *viride*, which contrasts with the finding that *A. atkinsonii*, *A. rupestre*, and members of clades III and IV are all diploids (Takamiya 1996). Speciation at the polyploid level appears to be unlikely due to the existence of *A. frangulum* f. *viride*, but any selective pressure for the success of polyploids may be related to the evolution of clade V.

Among the morphologically defined groups (Fig. 5), the *Athyrium otophorum* group *sensu* Kurata (1961) and section *Otophora* subsection *Otophora sensu* Wang (1997) could be referred to as clade Vb. *Athyrium arisanense* of the *A. otophorum* group is included in clade V, but its position varies among DNA datasets. The *Athyrium vidalii* group *sensu* Kurata (1961) was divided into two clades: *A. wardii* and *A. clivicola* were included in clade Vb with the members of the *A. otophorum* group, and *A. vidalii* was included in clade Va with *A. deltoidofrons* of the *A. filix-femina* group. In comparison, Wang (1997) classified *A. wardii* and *A. clivicola* in sect. *Otophora* subsect. *Otophora*, and classified *A. vidalii* in sect. *Mackinnoniana*, which includes *A. deltoidofrons*. Our phylogenetic analysis supports Wang's (1997) system with respect to this group of species.

The *Athyrium iseanum* group *sensu* Serizawa

(1981) is comparable to sect. *Otophora* subsect. *Strigillosa sensu* Wang (1997). As for this group of species, *A. iseanum*, *A. iseanum* var. *angustisectum*, and *A. strigillosum* form a clade in the *trnL–trnF* and combined dataset trees, but it is supported only by the Bayesian method. Therefore, the monophyly of this group cannot be confirmed in the present study.

The *Athyrium yokoscense* group *sensu* Tagawa (1933) corresponds to our clade IV, although the position of *A. rupestre* in the tree was uncertain. Our results do not support the taxonomic treatment of *A. yokoscense* by Wang (1987), in which it was grouped with *A. niponicum*. Nakato (1988) reported a somatic chromosome number of $2n = 78$ for *A. nikkoense*, and considered that the plants examined were derived by aneuploid reduction from $2n = 80$, which is common to the diploid species of *Athyrium* (Takamiya 1996). Recently, Takamiya *et al.* (in preparation) reexamined the chromosome number of the *A. yokoscense* group, and showed that *A. yokoscense, A. yokoscense* var. *dilatatum*, *A. tashiroi*, and *A. kirisimaense*, as well as *A. nikkoense*, had $2n = 78$, while *A. rupestre* had $2n = 80$. Therefore, clade IV may be a natural group having a derived basic chromosome number of $x = 39$. *Athyrium rupestre* has been treated as an ally of *A. yokoscense* (Tagawa 1933), but could not be related to *A. yokoscense.*

Finally, clade III corresponds to sect. *Athyrium sensu* Wang (1997). The *Athyrium filix-femina* group *sensu* Kurata (1961) should be redefined by deleting *A. deltoidofrons* and related taxa, which are more closely related to *A. vidalii.* Besides the groups listed in Fig. 5, there are some proposed intrageneric classifications in *Athyrium*. Kato (1977) divided the genus into two groups: the *A. puncticaule* group and the *A. filix-femina* group. Of the species used in this study, *A. atkinsonii* and *A. nakanoi* are included in the former group and the other species are included in the latter. The chloroplast DNA trees do not support this grouping because *A. nakanoi* is included in clade V while *A. atkinsonii* is not.

The PCR-SSCP analysis provides clues to the origin of hybrid species. *Athyrium oblitescens* is a fertile species, but its hybrid origin has been suspected based on a strong resemblance to *A.* ×*agipedis* Kurata, a sterile hybrid between *A. otophorum* and *A. wardii.* Serizawa (1980) pointed out subsequently that some of the plants identified as *A. oblitescens* were more similar to *A.* ×*purpureipes* Kurata, a sterile hybrid between *A. clivicola* and *A. otophorum*. Kurihara *et al.* (1996) examined the origin of *A. oblitescens* using allozyme analysis and chromosome counts and discovered that *A. oblitescens* comrpises different entities that originated independently from hybridization between *A. otophorum* and *A. wardii*, and between *A. otophorum* and *A. clivicola*. The chloroplast phylogenetic tree showed *A. oblitescens* together with *A. otophorum* and indicated that *A. otophorum* was the maternal ancestor. The SSCP band patterns, however, did not clearly determine whether *A. wardii* or *A. clivicola* was the paternal paretn in this sample. *Athyrium setuligerum* is morphologically intermediate between *A. clivicola* and *A. iseanum*, but it has normal spores, indicating fertility (Kurata 1966). Kurihara *et al.* (1996) presumed that *A. setuligerum* also was of hybrid origin as suggested in *A. oblitescens.* The chloroplast DNA sequence of *A. setuligerum* was identical to that of *A. clivicola* (Fig. 3), and the PCR-SSCP of *A. setuligerum* clearly showed the genetic contribution of *A. iseanum* (Fig. 4). Our analysis therefore showed that *A. clivicola* is the maternal ancestor and *A. iseanum* is the paternal ancestor. Furthermore, we were able to assess the parentage for all hybrids examined. The combination of chloroplast and nuclear DNA has proved to be a powerful method for detecting hybrid origins and resolving reticulate relationships in *Athyrium*, as was shown in other fern lineages (Ebihara *et al.*

2005, Adjie *et al.* 2007).

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APPENDIX 1. Species included in this study, source of materials, and GenBank accession number for the two chloroplast regions studied. Samples of which DNA sequences were determined were in boldface, and other samples were used only for PCR-SSCP of *PgiC*.

| Species and accession number | Source | *rbcL* | *trnL–trnF* |
|---|---|---|---|
| *Athyrium arisanense* (Hayata) Tagawa | | EU329025 | EU329069 |
| ***TD&T681*** | Yakushima, Kagoshima | | |
| *Athyrium atkinsonii* Bedd. | | EU329026 | EU329070 |
| ***BA603***, *BA604* | Arimine, Toyama | | |
| *Athyrium brevifrons* Nakai ex Kitag. | | EU329027 | EU329071 |
| *BA534, BA535,* ***BA536***, *BA537* | Abira, Hokkaido | | |
| *Athyrium clivicola* Tagawa | | EU329028 | EU329072 |
| ***BA528*** | Mt. Iwawaki, Osaka | | |
| ***BA533*** | Mt. Makio, Osaka | | |
| ***BA542*** | Kawachi, Osaka | | |
| ***BA547*** | Izumi, Osaka | | |
| *BA564* | Itsusuji, Osaka | | |
| *BA585* | Katoratani, Osaka | | |
| *BA587* | Mt. Kongo, Osaka | | |
| *BA596* | Takahatadani, Osaka | | |
| *BA601, BA602* | Bijodaira, Toyama | | |
| *BA617* | Sennan, Osaka | | |
| *Athyrium cuspidatum* (Bedd.) M.Kato | | EU329029 | EU329073 |
| ***BA630*** | Kunming Bot. Gard., China | | |
| *Athyrium deltoidofrons* Makino | | EU329030 | EU329074 |
| ***TBG122832*** | Tsukuba Botanic Garden | | |
| *BA567* | Sapporo, Hokkaido | | |
| *BA605* | Ranjou, Toyama | | |
| *BA611* | Azenotani, Osaka | | |
| *BA623* | Kisiwada, Osaka | | |
| ***BA625, BA626*** | Ootsujiyama, Toyama | | |
| *BA627* | Takayama, Toyama | | |
| ***MT6081607*** | Kuju, Ooita | | |
| *Athyrium eremicola* Oka & Sa.Kurata | | EU329031 | EU329075 |
| ***BA505*** | Hyogo Science Museum | | |
| *Athyrium filix-femina* (L.) Roth 'fezelliae' | | EU329032 | EU329076 |
| ***BA523*** | Cultivated | | |
| *Athyrium frangulum* Tagawa f. *viride* Sa.Kurata | | EU329033 | - |
| ***SM060806-8*** | Tsukuba Botanic Garden | | |
| *Athyrium iseanum* Rosenst. | | EU329034 | EU329077 |
| ***BA526*** | Mt. Iwaki, Osaka | | |

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|---|---|---|---|
| *BA530* | Mt. Makio, Osaka | | |
| *BA544* | Kawachi, Osaka | | |
| *BA549* | Izumi, Osaka | | |
| *BA560* | Itsutsuji, Osaka | | |
| *BA615, BA622* | Sennan, Osaka | | |
| *BA524* | Kisiwada, Osaka | | |
| *Athyrium iseanum* Rosenst. var. *angustisectum* Tagawa | | EU329035 | EU329078 |
| ***TD&T91*** | Hitoyoshi, Kumamoto | | |
| ***MT05112201*** | Yakushima, Kagoshima | | |
| *Athyrium kirisimaense* Tagawa | | EU329036 | EU329079 |
| *TS&T69* | Kirishima, Kagoshima | | |
| ***TD&T145*** | Karakuni, Miyazaki | | |
| ***TS&T106*** | Yakushima, Kagoshima | | |
| *Athyrium kuratae* Seriz. | | EU329037 | EU329080 |
| ***SM060806-9*** | Tsukuba Botanic Garden | | |
| ***MT6061202*** | Minamata, Kumamoto | | |
| ***MT6061204*** | Ookuchi, Kagoshima | | |
| *Athyrium melanolepis* (Franch. & Sav.) H.Christ | | EU329038 | EU329081 |
| ***BA501*** | Nikko Botanical Garden | | |
| *Athyrium nakanoi* Makino | | EU329039 | EU329082 |
| ***MT05112301*** | Yakushima, Kagoshima | | |
| *Athyrium neglectum* Seriz. subsp. *australe* Seriz. | | EU329040 | EU329083 |
| ***MT6081606*** | Kuju, Ooita | | |
| *Athyrium nikkoense* Makino | | EU329041 | EU329084 |
| ***TS&T170*** | Fujimi, Shizuoka | | |
| *Athyrium niponicum* (Mett.) Hance | | EU329042 | EU329085 |
| ***BA506***, *BA507* | Yayoi, Chiba | | |
| *Athyrium oblitescens* Sa.Kurata | | EU329043 | EU329086 |
| ***BA579*** | Inazumi, Toyama | | |
| ***BA598*** | Kamikage, Hyogo | | |
| *Athyrium otophorum* (Miq.) Koidz. | | EU329044 | EU329087 |
| SM060806-30 | Tsukuba Botanic Garden | | |
| ***TD&T134, TD&T382*** | Minamata, Kumamoto | | |
| ***TD382*** | Kagoshima, Kagoshima | | |
| ***TD&T162*** | Mt. Kurama, Kyoto | | |
| ***BA548*** | Izumi, Osaka | | |
| *BA566* | Uenokumi, Osaka | | |
| *BA613* | Azenotani, Osaka | | |
| *BA618, BA619* | Sennan, Osaka | | |
| *Athyrium rupestre* Kodama | | EU329045 | EU329088 |
| ***BA539*** | Sapporo, Hokkaido | | |
| ***TS&T510, TS&T511*** | Ojika, Akita | | |
| *Athyrium setuligerum* Sa.Kurata | | EU329046 | EU329089 |
| ***MT6081606*** | Kuju, Ooita | | |
| *Athyrium sheareri* (Baker) Ching | | EU329047 | EU329090 |
| ***BA522*** | Yayoi, Chiba | | |
| *Athyrium subrigescens* Hayata ex H.Itô | | EU329048 | EU329091 |
| ***TD&T138*** | Itsuki, Kumamoto | | |
| ***TD&T413*** | Yakushima, Kagoshima | | |

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| *Athyrium strigillosum* T. Moore ex Salomon | | EU329049 | EU329092 |
| ***MT6081602*** | Teno, Kumamoto | | |
| *Athyrium tashiroi* Tagawa | | EU329050 | EU329093 |
| ***TS&T83*** | Mt. Hagane, Fukuoka | | |
| ***TS&T79*** | Mt. Rai, Fukuoka | | |
| ***TS&T97***, *TS&T99* | Mt. Hiko, Fukuoka | | |
| *Athyrium tozanense* Hayata | | EU329051 | EU329094 |
| ***MT05112302*** | Yakushima, Kagoshima | | |
| *Athyrium vidalii* (Franch. & Sav.) Nakai | | EU329052 | EU329095 |
| ***TBG137195*** | Tsukuba Botanic Garden | | |
| *BA521* | Yayoi, Chiba | | |
| ***BA525*** | Mt. Iwawaki, Osaka | | |
| *BA529* | Mt. Makio, Osaka | | |
| *BA543* | Kawachi, Osaka | | |
| *BA558, BA559* | Itsutsuji, Osaka | | |
| ***BA583*** | Kurotsugatani, Osaka | | |
| *BA584* | Katoratani, Osaka | | |
| ***BA589*** | Mt. Kongo, Osaka | | |
| *BA595* | Takahatadani, Osaka | | |
| ***BA608*** | Takakura, Osaka | | |
| *BA612* | Azenotani, Osaka | | |
| ***BA614*** | Sennan, Osaka | | |
| *BA568, BA571* | Ooiwa, Toyama | | |
| *BA551* | Jozankei, Hokkaido | | |
| *Athyrium viridescentipes* Sa.Kurata | | EU329053 | EU329096 |
| ***TD&T153*** | Ookuchi, Kagoshima | | |
| ***TD&T164*** | Fukuoka, Fukuoka | | |
| *Athyrium wardii* (Hook.) Makino | | EU329054 | EU329097 |
| ***BA527*** | Mt. Iwawaki, Osaka | | |
| ***BA531*** | Mt. Makio, Osaka | | |
| *BA550* | Izumi, Osaka | | |
| *BA562* | Itsutsuji, Osaka | | |
| *BA582* | Kurotsugatani, Osaka | | |
| *BA586* | Katoratani, Osaka | | |
| *BA606* | Takakura, Osaka | | |
| *BA616, BA620* | Sennan, Osaka | | |
| *Athyrium yokoscense* (Franch. & Sav.) H.Christ | | EU329055 | EU329098 |
| ***BA500*** | Mt. Apoi, Hokkaido | | |
| *BA524* | Mt. Iwawaki, Osaka | | |
| ***BA532*** | Mt. Makio, Osaka | | |
| *BA540* | Kawachi, Osaka | | |
| *BA546* | Izumi, Osaka | | |
| *BA561* | Itsutsuji, Osaka | | |
| *BA565* | Uenokumi, Osaka | | |
| *BA590* | Mt. Kongo, Osaka | | |
| *BA597* | Takahatadani, Osaka | | |
| *BA552, BA553, BA554, BA555, BA556* | Tomakuma, Hokkaido | | |
| *BA600* | Arimine, Toyama | | |
| ***TD&T179*** | Mt. Tsurugi, Tokushima | | |

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|---|---|---|---|
| *Athyrium yokoscense* var. *dilatatum* Tagawa | | EU329056 | EU329099 |
| ***TS&T19*** | Mt. Tsurumi, Ooita | | |
| ***TS&T55*** | Kuju, Ooita | | |
| ***TD&T154*** | Mt. Tsurugi, Tokushima | | |
| *Athyrium* ×*akiense* Sa.Kurata | | EU329057 | EU329100 |
| ***BA516*** | Koishikawa Bot. Garden | | |
| *Athyrium* ×*hisatsuanum* Sa.Kurata | | EU329058 | EU329101 |
| ***BA517*** | Koishikawa Bot. Garden | | |
| *Athyrium* ×*tokashikii* Sa.Kurata | | EU329059 | EU329102 |
| ***BA563*** | Itsutsuji, Osaka | | |
| *Cornopteris christenseniana* (Koidz.) Tagawa | | | |
| ***BA508*** | Koishikawa Bot. Garden | EU329060 | EU329103 |
| ***BA592*** | Mt. Kongo, Osaka | EU329061 | EU329104 |
| *Cornopteris crenulatoserrulata* (Makino) Nakai | | EU329062 | EU329105 |
| ***BA509*** | Koishikawa Bot. Garden | | |
| *Cornopteris decurrenti-alata* (Hook) Nakai | | EU329063 | EU329106 |
| ***BA511*** | Koishikawa Bot. Garden | | |
| *Deparia petersenii* (Kunze) M.Kato | | EU329064 | EU329107 |
| ***BA520*** | Koishikawa Bot. Garden | | |
| *Deparia conilii* (Franch. & Sav.) M.Kato | | EU329065 | EU329108 |
| ***BA518*** | Koishikawa Bot. Garden | | |
| *Deparia lobatocrenata* (Tagawa) M.Kato | | EU329066 | EU329109 |
| ***BA519*** | Koishikawa Bot. Garden | | |
| *Diplazium nipponicum* Tagawa | | EU329067 | EU329110 |
| ***BA512*** | Koishikawa Bot. Garden | | |
| *Diplazium hachijoense* Nakai | | EU329068 | EU329111 |
| ***BA513*** | Koishikawa Bot. Garden | | |

The specimens with prefix TD&T, TS&T and MT were deposited at Kumamoto University (KUMA), those prefixed BA were deposited at Chiba University, and those prefixed TBG and SM were deposited at Tsukuba Botanic Garden.